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Calreticulin promotes angiogenesis via activating nitric oxide signalling pathway in rheumatoid arthritis

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Summary

Calreticulin (CRT) is a multi-functional endoplasmic reticulum protein implicated in the pathogenesis of rheumatoid arthritis (RA). The present study was undertaken to determine whether CRT was involved in angiogenesis via the activating nitric oxide (NO) signalling pathway. We explored the profile of CRT expression in RA (including serum, synovial fluid and synovial tissue). In order to investigate the role of CRT on angiogenesis, human umbilical vein endothelial cells (HUVECs) were isolated and cultured in this study for *in-vitro* experiments. Our results showed a significantly higher concentration of CRT in serum (5·4 ± 2·2 ng/ml) of RA patients compared to that of osteoarthritis (OA, 3·6 ± 0·9 ng/ml, $P < 0·05$) and healthy controls (HC, 3·7 ± 0·6 ng/ml, $P < 0·05$); and significantly higher CRT in synovial fluid (5·8 ± 1·2 ng/ml) of RA *versus* OA (3·7 ± 0·3 ng/ml, $P < 0·05$). High levels of CRT are expressed in synovial membrane localized predominantly to inflammatory cells and synovial perivascular areas in both the lining and sublining layers of RA synovial tissue (RAST). Increased nitric oxide (NO) production and phosphorylation level of endothelial nitric oxide synthase (eNOS) were measured in HUVECs following CRT stimulation, while the total eNOS expression was not significantly changed. Furthermore, CRT promoted the proliferation, migration and tube formation of HUVECs, which were significantly inhibited by a specific eNOS inhibitor. These findings suggested that CRT may be involved in angiogenesis events in RA through NO signalling pathways, which may provide a potential therapeutic target in the treatment of RA.

Keywords: angiogenesis, calreticulin, nitric oxide, rheumatoid arthritis

Introduction

Calreticulin (CRT) is a multi-functional chaperone in the endoplasmic reticulum (ER), which facilitates Ca^{2+} homeostasis and the folding of major histocompatibility complex (MHC) class I molecules, thereby influencing antigen presentation to T cells [1]. Intracellular CRT can be secreted from cells and expressed on the cell surface or in the extracellular environment [2]. Cell surface CRT (csCRT), exposed mainly on apoptotic cells and human cancer cells, is relevant to phagocytic uptake and immunogenicity of cells [3–5]. CRT was shown previously to be a B cell molecular target in some gastrointestinal malignancies and may contribute to anti-cancer immunotherapy [6]. Extracellular CRT has been detected in the body fluids of patients with autoimmune disease, such as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) [7,8]. Recent studies indicated that markedly increased extracellular CRT in serum correlated with disease activity in patients with RA [7,9].

RA is a chronic autoimmune disease characterized by synovial inflammation and the formation of invading pannus [10]. Rheumatoid pannus, which leads to the destruction of articular cartilage and bone, has been referred to as 'aggressive, invasive, proliferative and tumor-like' [11,12]. Angiogenesis, the principal components of invading pannus in RA, acts as both a mediator for the recruitment of peripheral leucocytes and a source of nourishment [13,14]. The proliferation, migration and tube formation of endothelial cells are considered to play a key role

Table 1. Clinical characteristics of the patients in each group.

Group *n*	Gender(F/M)	Age(years)	CRP(mg/l)	ESR(mm/h)
RA 85	60/25	57 ± 12	58·8 ± 34·9*	48·6 ± 14·2*
OA 70	54/16	56 ± 13	31·2 ± 19·2	24·3 ± 17·8
HC 75	55/20	53 ± 8	6·6 ± 3·1	12·1 ± 10·9

*$P < 0{\cdot}05$ RA *versus* OA, RA *versus* HC. RA = rheumatoid arthritis; OA = osteoarthritis; HC = healthy control; CRP = C-reactive protein; ESR = erythrocyte sedimentation rate. Values are mean ± standard deviation.

in the process of angiogenesis [15]. Previous studies indicated that proinflammatory factors were involved in the angiogenesis and pannus formation in RA, which may be performed through stimulation on the proliferation, migration and tube formation of endothelial cells.

Nitric oxide (NO), synthesized from L-arginine by NO synthetases (NOS_S), is a ubiquitous signalling molecule with versatile functions in the immune system [16]. The proinflammatory effects of NO in RA have been reported, as indicated by the results that increased NO levels correlated significantly with disease activity and inflammatory markers of the disease [17,18]. Moreover, anti-rheumatic drugs have been shown to inhibit the production of NO [19]. Ling *et al.* [20] demonstrated that the RA shared epitope [a five amino acid sequence motif in position 70–74 of the human leucocyte antigen D-related (HLA-DR) β-chain encoded by HLA-DRB1 alleles which are strongly associated with susceptibility to severe RA] acts as a trigger for increased production of NO, which leads to higher susceptibility to oxidative DNA damage. A number of studies have identified that NO contributes significantly to the proangiogenic programme of capillary endothelium [21,22] and vascular endothelial growth factor (VEGF), a major regulator of vasculogenesis and angiogenesis, induces proliferation and angiogenesis in an NO-dependent manner [23]. NO may represent a promising target for pro- and anti-angiogenic therapeutic strategies.

Multiple lines of evidence indicated that CRT may be involved in RA and associated with NO production. A previous study by Tarr *et al.* [7] found that extracellular CRT was increased in the joints of patients with RA and inhibited FasL-mediated apoptosis of T cells. Furthermore, recent evidence indicated that csCRT played a critical role in RA by transducing shared epitope activated NO prooxidative signalling and T helper type 17 (Th17)-polarizing signals [24,25]. In addition, over-expression of CRT was shown previously to increase NO production in some cell systems [26,27].

Although CRT has been shown previously to be associated with RA, the pathogenic roles of CRT in synovial inflammation and pannus formation remain unclear. Given the known association of the CRT with RA, its association with NO production and the link between NO production and angiogenesis, we sought to determine whether CRT is involved in angiogenesis and pannus formation via activating NO signalling events in RA.

Materials and methods

Patients and samples

Serum samples were obtained from 85 patients with RA, 70 osteoarthritis (OA) and 75 age- and gender-matched healthy controls (HC) (Table 1). Samples of synovial fluid (SF) and synovial membrane (SM) tissues were obtained from 20 patients with RA and 18 OA undergoing synovectomy. Serum and SF samples were collected and kept for 1 h at 4°C for clotting, then centrifuged at 1400 *g* for 10 min, immediately aliquoted and stored at −80°C. Synovial tissues were obtained and placed immediately in sterile RPMI-1640 supplemented with 10% fetal bovine serum (FBS). Synovial tissue sections (5 μm thick) were cryostat-cut and used for immunohistochemistry. All patients with RA fulfilled the American College of Rheumatology (ACR) 1987 criteria for RA [28] and all patients with OA fulfilled the ACR 1995 criteria for OA [29]. Local ethics approval was provided (by the Medical Ethics and Human Clinical Trial Committee of Tianjin Medical University) for all experiments, and informed consent was obtained from all subjects studied.

Cell culture

Primary human umbilical vein endothelial cells (HUVECs) were isolated from fresh human umbilical cords via collagenase digestion, and subsequently maintained in Dulbecco's modified Eagle's medium (DMEM) medium supplemented with 12% FBS at 37°C in an atmosphere of 5% CO_2. All HUVECs were used after no more than five passages.

Measurement of CRT levels in serum and SF

The concentrations of CRT in serum and SF were quantified by a sandwich enzyme-linked immunosorbent assay (ELISA) kit (Xin Yue Inc., Shanghai, China), according to the manufacturer's protocol. Briefly, antibody specific for CRT was precoated onto a 96-well microplate. Serum and synovial fluid samples were diluted 1:15, and triplicate 40-μl samples were added to the wells. After incubation at 37°C for 2 h, the liquid of each well was removed and added 10 μl of biotin-conjugated rabbit anti-human CRT antibodies, followed by incubation for 1 h at 37°C. The wells were then

washed and incubated with 50 µl of streptavidin-conjugated horseradish peroxidase (HRP) at 37°C for 1 h. After washing five times, chromogenic substrate 3,3′,5,5′-tetramethylbenzidine (TMB) was added. The reaction was developed for 10 min at 37°C in the dark and was then terminated by adding the stop solution. The plates were assessed by ELISA plate reader at 450 nm. The expression of CRT in serum was measured as described previously [9].

CRT expression on RA synovial tissue (RAST) by immunohistochemical analysis

Sections (5 µm thick) were cut with a Leica RM 2145 cryostat, placed on 10% polylysine-coated glass slides and dried in the calorstat at 60°C for more than 2 h before staining. Tissue sections were then dewaxed in xylene and hydrated through ethanol to water. The slides were treated with PBS containing 0·3% H_2O_2 for 30 min to inactivate endogenous peroxidase activity. After washing, the slides were incubated with 5% bovine serum albumin (BSA) for 1 h at room temperature. The slides were then incubated with a polyclonal rabbit antibody to human CRT (PA3-900; ThermoFisher Scientific, Waltham, MA, USA) at 1:1000 dilutions at 37°C for 1 h. A secondary HRP-conjugated goat anti-rabbit immunoglobulin (Ig)G (Bioword, St Louis Park, MN, USA) was added at 1:1200 dilutions for 1 h at 37°C, and colour was developed with 3, 3′-diaminobenzidine. The sections were counterstained with haematoxylin, dehydrated and mounted. The expression of CRT was observed using microscopy and the images were analysed by the BioMias 2000 Image analysis and processing system.

NO assay by the Griess reagent

NO production was measured using a NO assay kit (Beyotime Biotechnology, Shanghai, China), according to the manufacturer's protocol. Briefly, the supernatants of HUVECs treated with different concentrations of recombinant human CRT (0, 1, 5, 10, 50 ng/ml) developed by ProSpec-Tany TechnoGene (Rehovot, Israel) were added to the 96-well culture plate, followed by colour development with Griess reagents; the absorbance was measured at 540 nm with a luminometer plate reader. Tumour necrosis factor (TNF)-α (PeproTech, Rocky Hill, NJ, USA) at 20 ng/ml served as a positive control.

Real-time reverse transcription–polymerase chain reaction (RT–PCR) assessment of eNOS mRNA expression

HUVECs in 50–60% confluency were stimulated with CRT at various concentrations in complete DMEM medium. After incubation for 24 h, total cellular RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. RT was performed using RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Glen Burnie, MD, USA). Real-time PCR amplification was performed using the Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen, Carlsbad, CA, USA), following the manufacturer's protocol. The following primers were used for eNOS: 5′-GTGGCTGTCTGCATGGACCT-3′ (forward) and 5′-CCACGATGGTGACTTTGGCT-3′ (reverse), product size 121 base pairs (bp). Relative gene expression in each group was determined by the ΔΔCt method with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal control.

Western blot analysis

HUVECs were incubated with various concentrations of CRT for the indicated time-periods. The cells were lysed in lysis buffer containing protease inhibitor cocktail and phosphatase inhibitor cocktail (Roche, Mannheim, Germany). Cell lysates (50 µg protein) were separated by 8% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes (Millipore Co., Billerica, MA, USA) at 200 mA for 2 h at 4°C. The membranes were blocked with 5% BSA in Tris-buffered saline (TBS) with 0·1% Tween-20 (TBST) for 1 h at room temperature. The primary antibodies to P-eNOS (Ser1177), total eNOS and β-actin purchased from Cell Signaling Technology (Beverly, CA, USA) were diluted 1:1000 in 5% BSA/TBST, respectively, and incubated at 4°C overnight. After washing with TBST three times, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h at room temperature. After washing, the bands were detected using a Pro-light HRP Chemiluminescent Kit (Tiangen Biotech, Beijing, China).

Cell proliferation by MTT assay

HUVECs (100 µl) were seeded in 96-well plates at a density of 2×10^3 cells/well in complete DMEM medium until the cells reached 50–60% confluency. The cells were then stimulated with increasing concentrations of CRT (0–50 ng/ml) in DMEM medium containing 1% FBS in the presence or absence of a specific eNOS inhibitor NG-nitro-L-arginine methyl ester (L-NAME, 1 mmol/l) developed by Beyotime Biotechnology (Shanghai, China) for 48–72 h. VEGF (PeproTech) at 10 ng/ml served as a positive control. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (20 µl, 5 mg/ml) was added to each of the wells and incubated for 4 h at 37°C. Then dimethylsulphoxide (DMSO) (150 µl) was added to solubilize the formazan crystals. Absorbance at 490 nm was measured by an automated microplate reader.

Cell migration assay using scratch wound healing

The migration of the HUVECs was measured as described previously [30,31]. In brief, HUVECs were cultured in

6-well plates which were drawn on the back with parallel lines in advance. A confluent monolayer of HUVECs was wounded with sterile pipette tips to create a linear wound across the well and the cellular debris was removed by washing with PBS. The wounded monolayers were incubated with recombinant human CRT (10 ng/ml) in the presence or absence of L-NAME (1 mmol/l) in DMEM medium containing 1% FBS. VEGF served as a positive control. Representative photomicrographs were taken and the wound-healing rates were analysed using Image J software.

Tube formation assay

HUVECs tube formation was measured using a three-dimensional *in-vitro* model. Fifty μl of rat tail tendon collagen type I (Solarbio, Beijing, China), and 190 μl of HUVECs cell suspension ($2{\cdot}0 \times 10^5$ cells/ml) supplemented with recombinant human CRT (10 ng/ml) in the presence or absence of L-NAME (1 mmol/l) were added to 96-well culture plates and allowed to polymerize for 20 min at room temperature. After adding DMEM medium, the plates were incubated at 37°C in 5% CO_2 for 0–72 h. The cell morphology was visualized via inverted microscopy and photographed.

Statistical analysis

Data were presented as mean ± standard deviation (s.d.), and were processed with SPSS software version 11·0. (SPSS Inc., Chicago, IL, USA). Differences among groups were analysed with one-way analysis of variance (ANOVA). Student–Newman–Keuls test was used for a comparison between two groups. Values for $P < 0{\cdot}05$ were considered statistically significant.

Results

Clinical characteristics of the subjects

A total of 230 subjects were involved in the current study. The detailed clinical and laboratory information of these subjects is shown (Table 1). There were no significant differences among all groups with regard to age distribution ($P > 0{\cdot}05$). Serum levels of CRP and ESR were significantly higher ($P < 0{\cdot}05$) in RA patients compared with OA patients and HC groups.

Increased levels of CRT in serum and SF samples from RA patients

The levels of CRT in serum and SF were measured by ELISA. Data of CRT levels in serum from different groups were analysed by one-way ANOVA. The difference was statistically significant ($F = 38{\cdot}093$, $P < 0{\cdot}05$). Further, paired comparisons were performed using Student–Newman–Keuls test; our results showed that CRT levels in serum of patients with RA (5·4 ± 2·2 ng/ml) were significantly higher ($P < 0{\cdot}05$) compared with OA patients (3·6 ± 0·9 ng/ml) and HC (3·7 ± 0·6 ng/ml), and there were no significant differences between OA patients and HC groups. CRT levels in RA SF (5·8 ± 1·2 ng/ml) were significantly higher ($P < 0{\cdot}05$) compared with OA SF (3·7 ± 0·3 ng/ml), and there were no significant differences between serum CRT levels and SF CRT levels in patients with RA (Fig. 1a). Western blot results showed that one single protein brand, molecule weight 46 kDa, was identified in all groups. Increased CRT levels were detected in serum of RA compared with OA and HC (Fig. 1b).

Increased expression of CRT in RAST

To identify CRT expressed in RAST, immunohistochemical analysis was performed on tissue sections. The pathological results showed significant hyperplasia and hypertrophy of synovial cells, accumulation of inflammatory cells and extensive neovascularization within the inflamed synovium of RA visualized by haematoxylin and eosin (H&E) staining. The immunohistochemical results showed that CRT was detected in all RAST samples and with minimal expression in all OA tissue sections. Strong CRT staining was located in synovial vascular endothelial cells, inflammatory cells and perivascular areas in both the synovial lining and sublining layers of synovial tissues in RA patients, while weak staining in synovial lining layers and perivascular areas was found in OA tissue sections (Fig. 2). The images were analysed by the BioMias image analytical instrument; the integral absorbance values (IA) of RA patients (IA value: 30 630 ± 19 747) were significantly higher ($P < 0{\cdot}05$) compared with OA patients (IA value: 6914 ± 2663).

Increased NO production and eNOS phosphorylation followed by CRT stimulation

In order to identify whether CRT had an impact on NO production in HUVECs, NO production after treatment with CRT was measured. Significant increases of NO production in HUVECs were detected which were associated with increasing concentrations of CRT, e.g. in a concentration-dependent manner (Fig. 3).

The effect of CRT on expression and phosphorylation of eNOS in HUVECs was investigated with real-time PCR and Western blot analysis. The results showed that there was no significant effect of CRT on total eNOS expression in both mRNA and protein levels. However, increased phosphorylation levels of eNOS following CRT stimulation were observed in a concentration-dependent manner. As dramatically elevated phosphorylation eNOS was observed when CRT concentrations were at 5~10 ng/ml, a concentration of CRT 10 ng/ml was selected as stimulator for

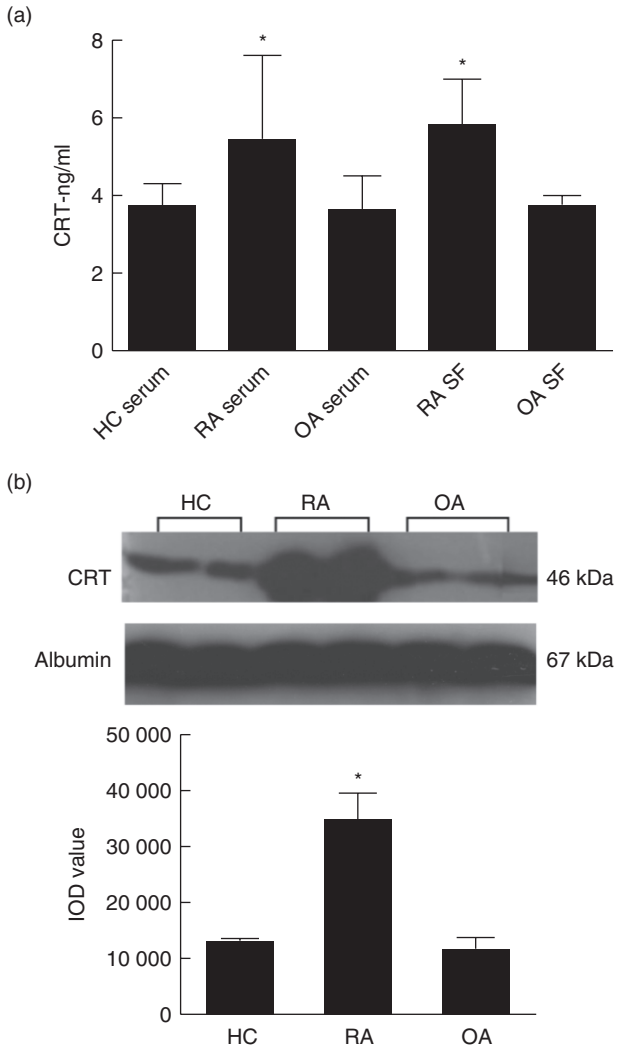

Fig. 1. Increased calreticulin (CRT) levels in rheumatoid arthritis (RA) patients. (a) Increased concentrations of CRT in serum and synovial fluid samples from RA patients. A sandwich enzyme-linked immunosorbent assay (ELISA) kit was used to measure the concentration of CRT in serum from healthy control (HC) subjects, and serum and synovial fluid (SF) samples from patients with RA and patients with osteoarthritis (OA). Values are expressed as mean ± standard deviation. *$P < 0{\cdot}05$ *versus* other groups. (b) Representative expression of CRT in serum of RA, OA and HC subjects by Western blot. One single protein brand, molecule weight 46 kDa, was identified in all groups. The expression of CRT was significantly higher in serum of patients with RA compared with OA and HC subjects (*$P < 0{\cdot}05$, $n = 15$).

HUVECs for 120 min. The results showed that increased eNOS phosphorylation was observed initially at 15 min after CRT treatment and reached the maximum at 120 min; increases of eNOS phosphorylation were in a time-dependent manner (Fig. 4).

CRT-induced proliferation, migration and tube formation of HUVECs

To analyse the impact of CRT on the proliferation of HUVECs, subconfluent HUVECs were treated with different concentrations of CRT (0–50 ng/ml) in the presence or absence of a specific eNOS inhibitor L-NAME for 48–72 h. Slight increases in HUVECs proliferation were observed with 1–5 ng/ml CRT. The increases were statistically significant when HUVECs were incubated with CRT concentration >10 ng/ml. The treatment of HUVECs with CRT induced increased cell numbers in a concentration-dependent manner, as determined by MTT assay. Moreover, the effects of CRT were inhibited significantly by co-stimulation with a specific eNOS inhibitor L-NAME (Fig. 5a).

Next, the role of CRT on the migration of HUVECs was determined by scratch wound-healing assay. Our results demonstrated a significant wound closure of HUVECs induced by CRT, which was comparable to that of VEGF (data not shown). Furthermore, this effect of CRT was decreased markedly in the presence of L-NAME. No significant effect on cell migration was observed with basal control or L-NAME alone. The migratory activity of the HUVECs stimulated with CRT or L-NAME was quantified (Fig. 5b).

Finally, a three-dimensional *in-vitro* tube formation assay was used to detect the effects of CRT on the morphological differentiation of HUVECs. Our results showed an enhanced formation of branched and capillary-like tube structures followed by CRT stimulation, which was inhibited significantly upon co-incubation with L-NAME. No significant effect was observed with basal control or L-NAME alone (Fig. 5c).

Discussion

RA is a progressive inflammatory autoimmune disease. Angiogenesis is a critical early event in synovial inflammation which facilitates a self-perpetuating infiltration of immune cells, and results in synovial hyperplasia and the destruction of adjacent cartilage and bone. The underlying mechanisms of angiogenesis have not yet been elucidated clearly. In the present study, we demonstrated increased expression of CRT in RA synovial tissues as well as in serum and SF. We also demonstrated increased NO production and phosphorylation levels of eNOS followed by CRT stimulation. In addition, we have shown that CRT significantly promoted proliferation, migration and tube formation of HUVECs, which were suppressed by eNOS inhibitor.

In the present study, elevated CRT levels were found in serum and SF of patients with RA compared with OA and HC. Our results were consistent with a recent study by Tarr *et al.* [7], which showed increased CRT levels in the plasma

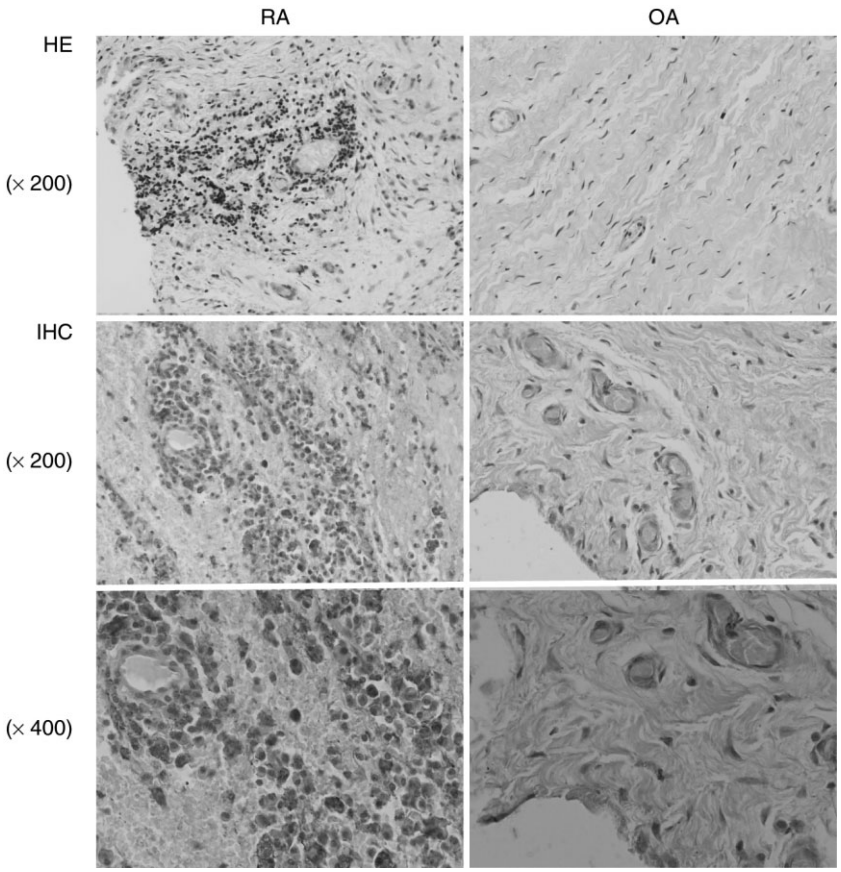

Fig. 2. Increased expression of calreticulin (CRT) in rheumatoid arthritis (RA) synovial tissues. The pathological results showed significant hyperplasia and hypertrophy of synovial cells, accumulation of inflammatory cells and extensive neovascularization within the inflamed synovium of RA visualized by haematoxylin and eosin (H&E) staining. Representative immunohistochemical results showed that CRT was expressed strongly in RA synovium localized to vascular endothelium, inflammatory cells and perivascular areas in both the lining and sublining layer regions. Weak staining in synovial lining layers and perivascular areas was observed in osteoarthritis (OA) synovial tissue.

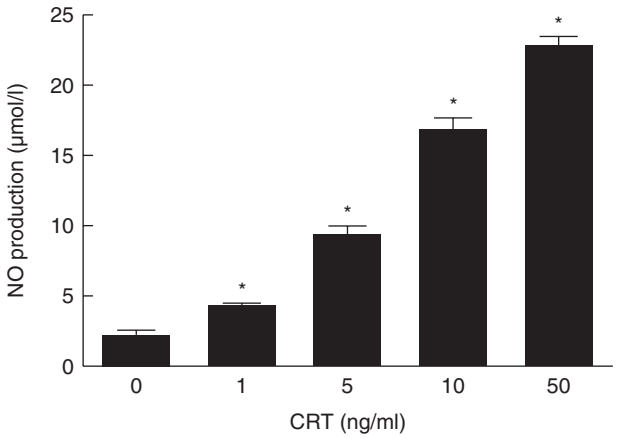

Fig. 3. Increased production of nitric oxide (NO) following calreticulin (CRT) stimulation. A NO assay kit was used to measure the production of NO by human umbilical vein endothelial cells (HUVECs) treated with graded concentrations of CRT (0–50 ng/ml). Tumour necrosis factor (TNF)-α (20 ng/ml) served as a positive control (data not shown). CRT stimulation for 24 h increased NO production in a concentration-dependent manner in HUVECs ($^*P < 0{\cdot}05$, $n = 5$).

and SF of patients with RA. Hong *et al.* [8] reported that serum CRT levels were higher in RA patients compared with SLE patients. Moreover, previous studies demonstrated a significant correlation between serum and SF CRT levels and disease activity in patients with RA [7,9]. The accumulated data suggested a pathogenic role for CRT in driving the proinflammatory events in RA. SF samples obtained from local damaged joints are related closely to the pathological state of the disease. Our results, together with previous studies, suggested that high circulating levels of CRT in RA serum may result from a high local production of CRT in the damaged joints. The concentrations of CRT in peripheral blood were comparable to those of CRT in local lesion sites, suggesting a beneficial effect of serum CRT for clinical management in RA.

We further investigated the expression of CRT in synovial tissues of RA. Our studies provide the first experimental evidence, to our knowledge, that the expression of CRT is increased in RA synovial tissues. The pathological results showed significant hyperplasia and hypertrophy of synovial cells, accumulation of inflammatory cells and extensive neovascularization within the inflamed synovium of RA. CRT was expressed highly in the synovial tissues of RA, and the synovial distribution of CRT corresponded closely to the regions of inflammatory cells accumulation and angiogenesis, while very low expression of CRT was found

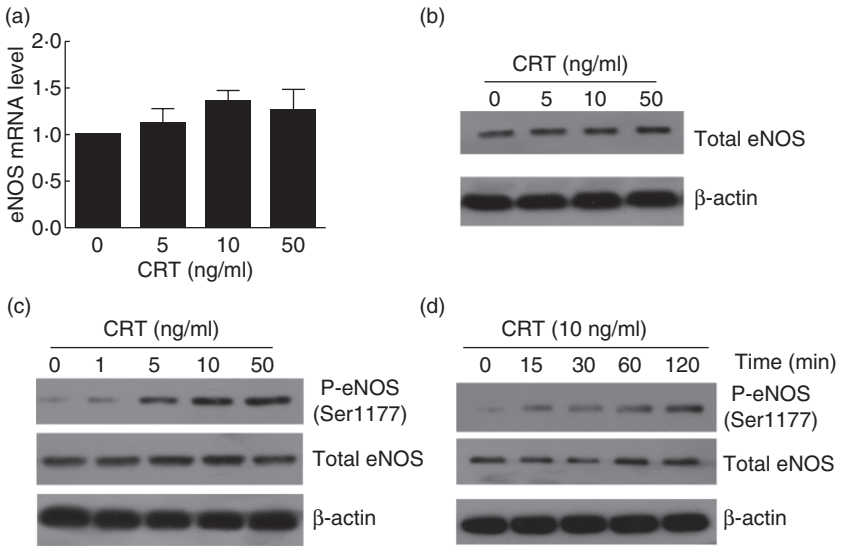

Fig. 4. Effect of calreticulin (CRT) on expression and phosphorylation of endothelial nitric oxide synthase (eNOS) in human umbilical vein endothelial cells (HUVECs). HUVECs in 50–60% confluency were incubated with 0–50 ng/ml CRT in complete Dulbecco's modified Eagle's medium (DMEM) for 24 h, real-time polymerase chain reaction (PCR) (a) and Western blot analysis (b) showed that CRT had no significant effect on eNOS expression in HUVECs. (c) HUVECs in 80–90% confluency were incubated with 0–50 ng/ml CRT for 2 h, Western blot analysis showed that CRT treatment dose-dependently increased phosphorylation levels of eNOS Ser1177 in HUVECs. (d) HUVECs in 80–90% confluency were incubated with 10 ng/ml CRT for the indicated time-periods; Western blot analysis showed increased phosphorylation levels of eNOS Ser1177 following CRT stimulation in a time-dependent manner.

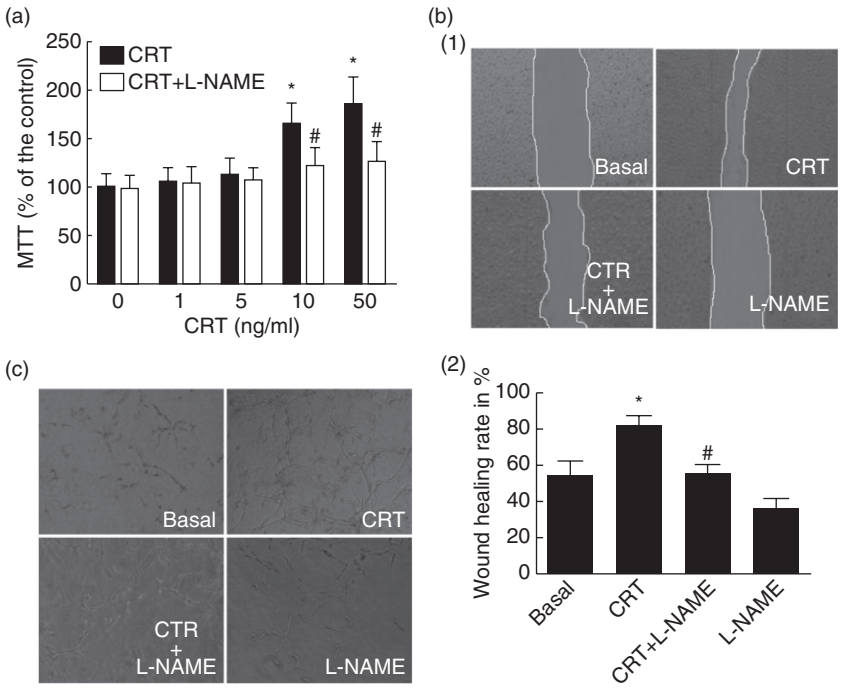

Fig. 5. Calreticulin (CRT)-induced proliferation, migration and tube formation of human umbilical vein endothelial cells (HUVECs). (a) HUVECs were stimulated with increasing concentrations of CRT (0–50 ng/ml) for 48–72 h and cell proliferation was measured by MTT assay. Significant increases in proliferation of HUVECs were showed with both 10–50 ng/ml CRT and vascular endothelial growth factor (VEGF) ($n = 5$). The data were expressed as % of control cells. VEGF (10 ng/ml) served as a positive control (data not shown). *$P < 0{\cdot}05$ *versus* control cells. CRT-induced proliferation of HUVECs was inhibited by co-stimulation with L-NAME. #$P < 0{\cdot}05$, CRT + NG-nitro-L-arginine methyl ester (L-NAME) *versus* CRT. (b1) The migration of HUVECs was significantly increased followed by CRT stimulation, as evidenced by cells repopulating the wound. CRT-induced migration of HUVECs was markedly inhibited upon CRT/L-NAME co-stimulation. (b2) Quantification of HUVECs migration ($n = 5$). The bar corresponded with b1. *$P < 0{\cdot}05$ *versus* basal control. #$P < 0{\cdot}05$, CRT + L-NAME *versus* CRT. (c) Representative photomicrograph showed HUVECs tube formation following incubation with CRT alone or in the presence of L-NAME. Enhanced formation of branched and capillary-like tube structures was observed followed by CRT stimulation, which was decreased upon coincubation with L-NAME.

in synovial tissues of OA. The expression levels and location of CRT were significantly different between RA and OA patients. The change in local tissues such as synovial tissues and SF can reflect the pathological state more accurately. The increased expression of CRT in local lesions of RA synovial tissues and SF suggested that CRT may have a direct pathogenic role in facilitating joint damage in RA.

In the present study, the role of CRT on NO production was investigated. We demonstrated increased NO production of HUVECs in response to CRT stimulation. Because eNOS plays a critical role in NO production, the levels of expression and phosphorylation of eNOS in HUVECs followed by CRT stimulation were examined further. Our results showed elevated phosphorylation levels of eNOS following CRT stimulation, while no significant effect on total eNOS expression was detected. As is well known, phosphorylation is considered to be one of the most important post-translational regulatory patterns on eNOS activity [32]. Our results indicated that CRT-induced phosphorylation of eNOS was mainly responsible for the elevated NO production in HUVECs. NO is a multifunctional signalling molecule that plays an important role in mediating cell functions at the sites of synovial inflammation in RA. NO functions as a second messenger that activates soluble guanyl cyclase and participates in signal transduction pathways involving cyclic guanosine monophosphate (GMP) [33]. NO was reported previously to be generated primarily by endothelial cells of synovial capillaries, osteoblasts, osteoclasts, fibroblasts, lymphocytes, neutrophils and macrophages in the inflamed synovium [33,34]. Overproduction of NO was demonstrated previously to contribute to T lymphocyte dysfunction in RA by altering multiple signalling pathways in T cells [35]. NO has been described to reinforce anti-apoptotic pathways in RA fibroblast-like synoviocytes [36–38]. Additionally, it is well documented that NO has a significant effect on angiogenesis [21–23,39,40]. The precise pathogenic mechanism of NO in RA needs to be studied further. Our preliminary results from the present study contribute to the possibility that CRT may play a role in the pathogenesis of RA via a NO-dependent mechanism.

Subsequently, the role of CRT on angiogenesis was examined. The present results showed that CRT induced proliferation of HUVECs in a dose-dependent manner. Moreover, CRT was demonstrated to have a direct effect on the migration of HUVECs, which was similar to that seen with VEGF, a primary angiogenic mediator relatively specific to endothelial cells [41,42]. CRT also played a significant role in promoting tube formation of HUVECs. In addition, the concentrations of CRT used in our *in-vitro* experiments were comparable with the levels of CRT measured in RA patients, suggesting that these effects of CRT were likely to occur *in vivo*. Angiogenesis, the formation of new capillaries from pre-existing vessels, is a complex and multi-step process that involves endothelial cell proliferation, migration, basement membrane degradation and new lumen organization [15]. Angiogenesis is an early and fundamental event in RA synovial inflammation and pannus formation. The perpetuation of angiogenesis, which acts as both a conduit for the recruitment of peripheral leucocytes and a source of oxygen and nutrients for tissue metabolism, is a prerequisite for the growth and survival of the invading pannus [13,14]. Moreover, we showed that the effects of CRT promoting proliferation, migration and tube formation of HUVECs were inhibited by a specific inhibitor of eNOS. Our results suggested a pathogenic role of CRT in promoting angiogenesis through the NO-mediated signalling pathway.

In this study, increased expression of CRT in RA patients, especially in synovial tissue, were demonstrated, supporting a potential pathogenic role of CRT in RA. We also demonstrated increased NO production as well as elevated levels of phosphorylation eNOS followed by CRT stimulation. Furthermore, CRT significantly promoted proliferation, migration and tube formation of HUVECs, which were inhibited by a specific inhibitor of eNOS. Together, these findings provided strong evidence for a new signalling pathway of CRT in stimulating angiogenesis via NO in RA.

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Disclosure

The authors declare no conflicts of interest.

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